

Diapause Response of Laboratory Reared and Native *Lygus hesperus* Knight (Hemiptera: Miridae)

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ABSTRACT Inconsistencies among results of previous studies on the induction of adult diapause in the western tarnished plant bug, *Lygus hesperus* Knight, could be either attributed to differences in the criteria being applied to assess diapause status or to differences in the sources of the bugs. To determine the cause of variation and to refine the criteria used to define diapause status, we obtained eggs from adults collected from the field and from long-term laboratory colonies. Concurrent experiments were conducted at two locations. *L. hesperus* were reared from egg to adulthood under photophases of either 10 or 14 h and at a constant temperature (26.6°C, Shafter, CA; 27.5°C, Maricopa, AZ). Adults were dissected at 10-d posteclosion to measure diapause status using both stringent and relaxed morphological criteria. Under both sets of criteria, offspring of field caught parents were more likely to enter diapause with short day exposure than those offspring from stock colonies. The relaxed criteria appeared appropriate for distinguishing diapause in males, whose reproductive organs can appear undeveloped after mating, although the stringent criteria appeared appropriate for females. Additional analyses were conducted to determine whether extended laboratory rearing influenced other behavioral or physiological traits. Bugs from stock colonies were more likely to mate, and to oviposit as virgins, than were bugs originating from the field. The results demonstrate a pronounced effect of colony rearing on *L. hesperus* physiology and behavior, and also have led to refinement of the criteria used to assess diapause status.

KEY WORDS western tarnished plant bug, *Lygus hesperus*, diapause, rearing

Survival during seasonally adverse environmental conditions is achieved in many insects by entering a state of diapause. Diapause is a preprogrammed, genetically controlled dormancy that can reduce feeding and reproductive development, and is normally induced by predictable token stimuli. In temperate climates photoperiod frequently serves this stimulating role, although its effects are often modified by temperature, condition of the host plant, and other factors (Tauber et al. 1986). Diapause is typically induced in a stage of development other than that in which it is expressed, and once induced tends to be irreversible unless specific developmental or environmental conditions are met (Denlinger et al. 2005). Because diapause is part of a survival strategy, it usually has ecological implications such as the ability to survive for extended periods without access to food. However, the accompanying

behavioral and physiological changes may leave insects susceptible to some ecologically- or culturally-based management tactics (McGuire et al. 2006). Development of novel management tactics targeting overwintering *L. hesperus* populations will require an acute understanding of the diapause phenomenon. However, producing a consistent supply of diapausing bugs needed for such research has been hampered by an incomplete understanding of the inducing stimuli and inconsistent criteria used in identifying developmental status.

Nymphs of the western tarnished plant bug, *Lygus hesperus* Knight, develop into diapausing adults when exposed to short photophases. Beards and Strong (1966) and Leigh (1966) reported that almost all field-collected nymphs developing under photophases of 10.5–11.5 h entered diapause, and there was a sharp reduction in the occurrence of diapause outside of this narrow range. Under constant temperatures in the laboratory, a wider range of photophases (7–12 h) was sufficient to induce a high incidence of diapause (Beards and Strong 1966). Spurgeon and Brent (2010) observed that *L. hesperus* nymphs taken from a stock colony and reared under a 10-h photophase exhibited a much lower incidence of diapause than was reported by Beards and Strong (1966), despite the similarity in

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laboratory conditions between the two studies. Although Spurgeon and Brent (2010) and Beards and Strong (1966) used different morphological criteria to distinguish diapause, the substantial difference in the incidence of diapause between the two studies suggests the involvement of additional factors. We hypothesized that selection pressures imposed by laboratory rearing may have caused the diapause response of lab-reared insects to diverge from that of native populations. Our objective was to test the hypothesis of a divergent diapause response corresponding to bug source (field collected, long-term laboratory colony).

Materials and Methods

Concurrent experiments were conducted at USDA, ARS laboratories at Maricopa, AZ and Shafter, CA. Both locations maintained laboratory colonies that have been under continuous culture for >5 yr with periodic introductions of field collected adults to sustain vigor. Additional native adults were collected from nearby alfalfa (*Medicago sativa* L.) at each location. At the Maricopa location, the laboratory colony and field collected adults were maintained in mixed-sex groups within 1890-ml waxed chipboard cups (Huhtamaki, De Soto, KS) held at 27.5–30°C under a 14-h photophase and provisioned with pods of green beans (*Phaseolus vulgaris* L.) and artificial diet (Debolt 1982) presented in packets of Parafilm M (Pechiney Plastic Packaging, Chicago, IL) (Patana 1982). The laboratory colony at Shafter was maintained at 27–31°C under a 14-h photophase whereas field collected adults were held at 26.6 ± 1°C. Adults were held in 0.03-m³ screened oviposition cages and colony-reared nymphs were held in 3.78-liter plastic buckets with screened lids. Oviposition cages and rearing buckets were provisioned with green bean pods and raw sunflower seeds (*Helianthus annuus* L.), and shredded paper was provided as a refuge.

Eggs were obtained from the respective laboratory colonies by placing fresh green bean pods within the rearing cages for 18–24 h. Eggs similarly were obtained from cages containing >500 adults recently collected from nearby alfalfa (*Medicago sativa* L.). Beans recovered from the rearing cages were cut in half, and each half was placed on a filter paper disk within a 100 × 15-mm petri plate. Petri plates were sealed with Parafilm M to prevent desiccation of the bean sections. Petri plates containing the bean sections were assigned to environmental chambers maintained at either a 10:14 or 14:10 (L:D) h photoperiod, ensuring that both halves of a given bean were not assigned to the same chamber. The chambers in Maricopa were kept at 27.5 ± 1°C, while those chambers at Shafter were held at 26.6 ± 1°C.

Bean sections were examined daily for newly-emerged nymphs beginning on the fourth day after oviposition. On the fifth day after oviposition, the parafilm was removed and a 4–5-cm-long section of fresh green bean pod was placed in each plate as a food

source for emerging nymphs. On the day of hatching, nymphs ($n = 50$) were transferred to their respective experimental containers and returned to the assigned photoperiod treatment. Nymphs held in mixed-sex groups at Maricopa were provided an *ad libitum* diet of green bean pods, which were replenished three times weekly or more often if the beans deteriorated. At Shafter, each nymph was held individually in an 18-ml plastic vial (Thornton Plastics, Salt Lake City, UT) closed with a screened snap-cap lid and provided a 4–5-cm section of green bean that was replaced three times weekly.

Beginning when fifth instars were first observed, nymphs were monitored daily to detect adult eclosion. The gender of each newly eclosed adult was determined based on the presence or absence of an ovipositor. At Maricopa, adults emerging on the same day were housed together in a mixed-sex group in a new container. At Shafter, the adults were kept in their individual vials, and the bean sections with which they were confined were examined for the presence of eggs on the day after adult eclosion and daily thereafter until the bug was dissected.

On the 10th day after adult eclosion each adult was dissected in saline (0.7% NaCl [wt:vol]) to determine whether it was in diapause. Adult diapause status was characterized based on the condition of the fat body and reproductive organs (Spurgeon 2009, Spurgeon and Brent 2010). The fat body was classified as being hypertrophied or not. A hypertrophied fat body was characterized by conspicuous deposits forming columns lateral to the dorsal vessel with globules of fat at the margins of the abdomen. Female reproductive state was classified according to a progressive scale of ovarian development: no development, vitellogenic oocytes present, or chorionated eggs present. In addition, the presence of follicular relics (indicative of recent ovipositional activity) was recorded. Male reproductive state was classified according to the conditions of the lateral and medial accessory glands. Lateral accessory glands were classified as undeveloped (containing no colored material) or developed (with colored material at the base of the gland and in the distal portion). Medial accessory glands were classified as undeveloped (small with no colored contents), underdeveloped (base contains some colored material but is not distended; distal section may or may not contain colored material but is not opaque), and developed (base filled or distended and distal section opaque with colored material).

Diapause status was recorded using two sets of criteria (stringent and relaxed) differing in the degree of reproductive development. Presence of a hypertrophied fat body was a prerequisite for a classification of diapause by either set of criteria. Females classified as diapausing exhibited no evidence of vitellogenesis under stringent criteria, but under relaxed criteria lacked only mature eggs or follicular relics. Males classed as diapausing contained undeveloped lateral and medial accessory glands by the stringent criteria, but included those males with underdeveloped medial glands using relaxed criteria.

In addition to diapause status, other characters of the bugs were recorded at dissection to determine whether their occurrence was associated with physiological status or bug origin. At the Maricopa location, bugs were held in mixed-sex groups. When females were dissected the occurrence and number of spermatophores present in the seminal depository were noted. At the Shafter location, the occurrence of a type of fat that is semitransparent and appears as a collection of glass beads (Spurgeon and Brent 2010) was noted. The experiments at each location were conducted three times.

Statistical Analyses. Under the 14-h photophase, quasi-complete separation caused by the small number of diapausing bugs at Maricopa precluded comparisons between photoperiods by logistic regression. Therefore, the association between photoperiod and diapause occurrence was examined in a contingency table controlling for adult gender and bug source. The association of diapause occurrence with photoperiod, based on counts within individual tables corresponding to combinations of bug gender and source, was examined using the two-sided Fisher Exact test (PROC FREQ, SAS Institute 2008). The overall association between diapause and photoperiod, controlling for bug source and gender, was tested using the Cochran–Mantel–Haenszel nonzero correlation statistic (Q_{CSMH} ; Stokes et al. 2000). Separate analyses were conducted for stringent and relaxed criteria of diapause. No bugs at Shafter entered diapause under the 14-h photophase so statistical tests comparing the incidence of diapause in response to 10- and 14-h daylengths were not possible.

Mixed-model logistic regression (PROC GLIMMIX, SAS Institute 2008) was used to examine the influences of bug gender and source on diapause response under the 10-h photophase. Separate analyses were conducted using stringent and relaxed diapause criteria at both locations. Fixed effects were bug gender and source (field, lab), and repetition of the experiment was used as a random effect.

Mating propensity under laboratory conditions was examined for bugs held in mixed gender groups at Maricopa. These examinations only included bugs reared under the 14-h photophase. The data were analyzed in contingency tables using the Fisher exact test (PROC FREQ, SAS Institute 2008). The analyses examined the association between bug source (field, lab) and counts of females categorized by the number of spermatophores in the seminal depository at the time of dissection.

For bugs reared individually at Shafter, the probability of oviposition by unmated females was compared between females originating from the field and those females from the lab colony. Females of field origin held under the 10-h photophase did not oviposit (nearly all entered diapause) and all females reared under the 14-h photophase were classed as reproductive regardless of bug source. Therefore, only the females reared under the 14-h daylength were included in the analyses. Mixed-model logistic regression (PROC GLIMMIX, SAS

Institute 2008) was used to examine the influence of bug source on the proportion of adult females ovipositing. Bug source (field, lab) was the only fixed effect, and repetition of the experiment was treated as a random effect.

Finally, frequency of occurrence of a recently described fat type appearing as minute glass beads (Spurgeon and Brent 2010) was examined. Low numbers of colony-reared bugs containing the glass bead fat precluded analysis of the whole data set by logistic regression because of quasi-complete separation. Therefore, the frequency of occurrence of the glass bead fat was compared between bug sources using the Cochran–Mantel–Haenszel nonzero correlation statistic controlling for bug gender and photoperiod. The frequency of occurrence of this fat body type in adults originating from the field was then compared between bug genders and photoperiods using logistic regression (PROC GLIMMIX, SAS Institute 2008). The mixed-model contained fixed effects of bug gender, photoperiod, and their interaction. Repetition of the experiment was used as a random effect.

Results

Photoperiod Influence on Diapause. Based on contingency tables controlling for bug gender and source (N ranged from 120 to 134) for the Maricopa location, two-sided Fisher exact tests indicated significant associations ($P < 0.01$) between diapause and photoperiod for either stringent or relaxed criteria. The nonzero correlation test of the overall hypothesis of association of diapause with photoperiod was significant using stringent ($Q_{CSMH} = 213.3$, $df = 1$, $P < 0.01$) or relaxed criteria ($Q_{CSMH} = 231.4$, $df = 1$, $P < 0.01$). Similar proportions of diapausing bugs were observed after exposure to 10-h photophases when either stringent (59% diapause) or relaxed criteria (63% diapause) were used. Under the 14-h photophase only 1% of bugs were diapausing regardless of the criteria used. At Shafter, no bugs reared under the 14-h photophase were classed as diapausing regardless of bug gender or source, so testing of an overall photoperiod effect was not possible.

Lygus Gender and Source Influence on Diapause. Logistic regression of the data collected from *Lygus* reared at Maricopa under the 10-h photophase and using the stringent diapause criteria indicated a significant influence of bug source on diapause status ($F = 37.27$; $df = 1, 8$; $P < 0.01$), but no influence of bug gender ($F = 3.73$; $df = 1, 8$; $P = 0.09$) and no source by gender interaction ($F = 0.31$; $df = 1, 8$; $P = 0.60$; Fig. 1A). The probability of diapause occurrence in bugs originating from the field (0.82 ± 0.08) was approximately twice that of bugs originating from the laboratory colony (0.40 ± 0.12). Although the probability of diapause in females (0.70 ± 0.11) was numerically higher than in males (0.57 ± 0.13), the difference was not statistically significant.

Use of the relaxed criteria to classify bugs reared at Maricopa resulted in modest increases in the observed incidence of diapause compared with the stringent

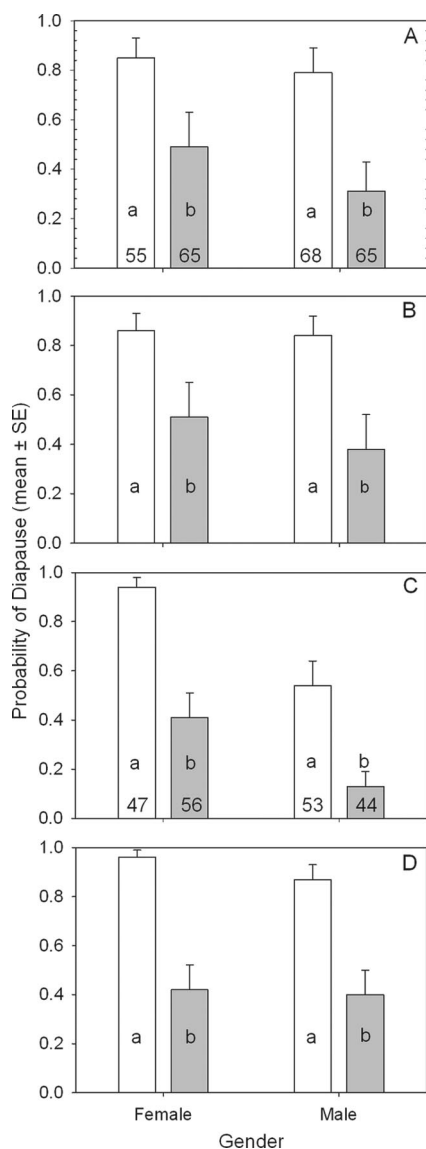


Fig. 1. Estimated probability (mean \pm SE) of occurrence of diapause in 10-d-old adult *L. hesperus* reared under a 10-h photophase. Bugs were obtained from the eggs of field collected adults (white bars) or from an established laboratory colony (gray bars) at two locations. Bugs were classified as diapausing or reproductive based on stringent (A) or relaxed criteria (B) at Maricopa, AZ, or by stringent (C) or relaxed criteria (D) at Shafter, CA. Different letters within the bars indicate significant effects of source on diapause status within gender. Numbers within bars indicate sample sizes.

criteria (Fig. 1B). However, effects indicated by the logistic regression were very similar to those effects observed for the stringent criteria. Using the relaxed criteria, the probability of entering diapause remained higher for bugs originating from the field (0.85 ± 0.07) than for colony reared bugs (0.44 ± 0.13 ; $F = 37.26$; $df = 1, 8$; $P < 0.01$). The probability of diapause

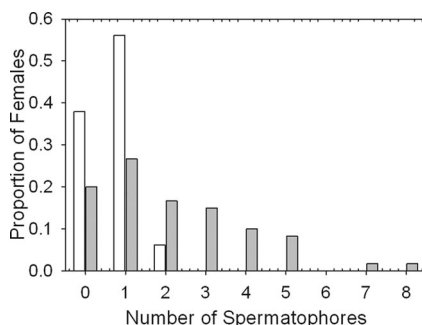


Fig. 2. Frequency distributions of numbers of spermatophores contained in 10-d-old adult female *L. hesperus* reared under a 14-h photophase at Maricopa, AZ. Bugs were obtained from the eggs of field collected adults (white bars, $n = 66$) or from an established laboratory colony (gray bars, $n = 60$).

occurrence did not differ between genders (females, 0.72 ± 0.11 ; males, 0.65 ± 0.13 ; $F = 1.25$; $df = 1, 8$; $P = 0.30$) and the bug gender by source interaction was not significant ($F = 0.33$; $df = 1, 8$; $P = 0.58$).

At Shafter, logistic regression of the data from individuals reared under a 10-h photophase and using the stringent diapause criteria indicated significant effects for both bug source ($F = 36.75$; $df = 1, 8$; $P < 0.01$) and gender ($F = 22.78$; $df = 1, 8$; $P < 0.01$). The nonsignificant gender by source interaction ($F = 1.38$; $df = 1, 8$; $P = 0.27$) suggested the respective influences of bug gender and source were consistent over levels of the other factor (Fig. 1C). In general, the probability of diapause occurrence was higher for females (0.77 ± 0.08) than for males (0.30 ± 0.08), and also higher for bugs originating from the field (0.81 ± 0.07) compared with those bugs from the laboratory colony (0.25 ± 0.07).

Analyses of data from Shafter using the relaxed diapause criteria indicated the probability of diapause in bugs reared from eggs of field-collected adults (0.92 ± 0.04) was higher than for bugs obtained from the colony (0.41 ± 0.09 ; $F = 38.17$; $df = 1, 8$; $P < 0.01$). As for the stringent criteria, the gender by source interaction was not significant ($F = 1.49$; $df = 1, 8$; $P = 0.26$). In contrast with analyses using the stringent criteria, under the relaxed criteria bug gender did not influence the probability of diapause (females, 0.80 ± 0.08 ; males, 0.68 ± 0.09 ; $F = 1.86$; $df = 1, 8$; $P = 0.21$). Compared with the stringent criteria, use of the relaxed criteria resulted in small increases in the occurrence of diapause in females, but substantial increases for males (Fig. 1D).

Lygus Source Influence on Mating Frequency. Contingency table analysis by using the Fisher exact test indicated a significant association of bug source with propensity to mate ($P < 0.01$) for females reared under the 14-h photophase at Maricopa (Fig. 2). Compared with bugs originating from the field, fewer females obtained from the laboratory colony were unmated by 10 d of age, and a higher proportion mated multiple times. The mean (\pm SE) numbers of sper-

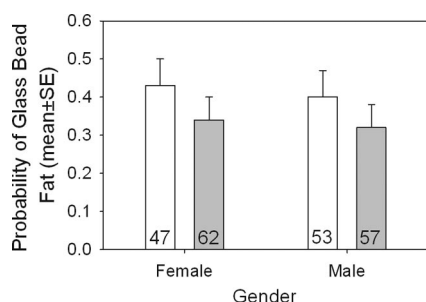


Fig. 3. Estimated probability (mean \pm SE) of occurrence of glass bead fat in 10-d-old adult *L. hesperus* reared under a 10- (white bars) or 14-h (gray bars) photophase at Shafter, CA. Numbers within bars indicate sample sizes.

matophores were 0.68 ± 0.24 for bugs originating from the field and 2.12 ± 0.24 for females obtained from the colony.

Lygus Source Influence on Oviposition by Virgin Females. Logistic regression indicated that at Shafter the source of bugs influenced the probability of oviposition by virgin females during the first 10 d of adulthood ($F = 12.38$; $df = 1, 4$; $P = 0.02$). The probability of oviposition by virgin females originating from the field (0.55 ± 0.15) was lower than for bugs obtained from the laboratory colony (0.95 ± 0.04).

Lygus Source Influence on Occurrence of 'Glass Bead' Type Fat. The nonzero correlation statistic examining the association of the glass bead fat type with bug source ($Q_{CSMH} = 82.6$; $df = 1$, $P < 0.01$) indicated the glass bead fat occurred more frequently in bugs originating from the field (37%, $n = 219$) than in those bugs obtained from the colony (0.5%, $n = 185$). Logistic regression examining only bugs obtained from the field did not indicate significant effects of photoperiod ($F = 1.63$; $df = 1, 1$; $P = 0.42$), bug gender ($F = 0.16$; $df = 1, 1$; $P = 0.76$), or their interaction ($F = 0.00$; $df = 1, 1$; $P = 0.98$; Fig. 3).

Discussion

Our results demonstrate that long-term laboratory rearing is associated with a variety of behavioral and physiological consequences that may hinder research on some aspects of *L. hesperus* basic biology. Of particular interest was the pronounced effect of bug source on diapause response to photoperiod (Fig. 1). Individuals of both genders originating from field collected adults were about twice as likely to enter diapause under the short-day rearing conditions as those individuals from the laboratory colony at Maricopa or Shafter. Spurgeon and Brent (2010) suggested the much lower incidence of diapause they observed for bugs reared under a 10-h photophase, compared with the responses in previous reports (Beards and Strong 1966, Leigh 1966, Strong et al. 1970), was likely caused in part by inconsistencies among the studies in the criteria used to distinguish diapause. However, Spurgeon and Brent (2010) also acknowledged

the possibility that bug source contributed to variation in results because some of the previous reports were based on field-collected insects. Results of our study confirm the importance of bug origin as a major source of variation in studies of *L. hesperus* diapause.

Although we demonstrated that long-term laboratory rearing can alter the diapause response of *L. hesperus*, continued concern for inconsistent use of diapause criteria among studies seems warranted. When the stringent diapause criteria were used at Shafter the observed incidence of diapause was significantly higher for females than for males, irrespective of bug source (Fig. 1C). Application of the relaxed criteria eliminated these differences (Fig. 1D). Unless an ecological reason exists for a different diapause response in males and females, these results suggest the stringent criteria may not be wholly sufficient for reliable detection of diapause in males. Similar differences in diapause response between genders, based on the stringent criteria, were not demonstrated at Maricopa, and relaxing of the distinguishing criteria resulted in comparatively modest increases in the apparent diapause response. Both stringent and relaxed criteria are somewhat subjective, especially for males. The different responses observed at Maricopa and Shafter may have been caused at least in part by differences in application of the criteria. In addition, the bugs at Maricopa were held in mixed-sex groups which allowed mating, whereas the bugs at Shafter were held singly. Therefore, access to a mate could have changed the perceived state of the male's reproductive organs. During mating the accessory glands are emptied, and may continue to appear underdeveloped at 24 h after mating (Brent 2010b). Also, a substantial proportion of reproductive bugs may exhibit a hypertrophied fat body (Spurgeon and Brent 2010). Therefore, the possibility exists that some recently mated reproductive males may have been classified as diapausing. Such misclassification might also explain the modest increases in apparent levels of diapause that were observed when the relaxed criteria were substituted for stringent criteria at Maricopa. The potential for such misclassification errors should be assessed through further study.

The subjective nature of the medial accessory gland ratings predisposes them to mis-application. Minimization of the potential for inconsistent use of these criteria warrants more specific descriptions of the classifications. Accordingly, we propose the following descriptions of medial accessory gland classes: 1) Not developed; deposits of white material in the basal section are distinctly cone-shaped, do not contact the walls of the gland lumen, and extend \leq one-half the length of the basal section. The basal section of the gland is not elongated and typically does not extend to the anterior margin of the bend in the seminal vesicles. The distal section of the gland is devoid of visible contents; 2) Underdeveloped; deposits of white material in the basal section extend $>$ one-half the length of the basal section of the gland. The deposits may be distinctly cone-shaped or may be rounded at the apex,

but do not contact the walls of the lumen. The basal section of the gland does not extend beyond the anterior margin of the bend in the seminal vesicles, and the distal section may contain a trace of white material but does not contain distinct opaque deposits; 3) Reproductive; deposits of white material in the basal section of the medial accessory glands generally fill the gland lumen, or if not, extend the length of the basal section. The basal section of the gland is obviously elongated and extends well past the bend in the seminal vesicles. The distal section of the gland contains opaque white deposits. In recently mated males, the contents of the basal and distal sections of the medial glands may be depleted of contents. However, the basal section of the gland is enlarged and extends well beyond the bend in the seminal vesicles. Use of these new descriptions, as well as continued examination of diapause using both stringent and relaxed criteria, should preserve the usefulness of observations regarding diapause until endocrinological evidence becomes available to more clearly distinguish *Lygus* physiological status.

Regardless of the diapause-distinguishing criteria we used, our estimates of the occurrence of diapause were generally lower than those estimates from previous reports (Beards and Strong 1966, Leigh 1966, Strong et al. 1970). As suggested by Spurgeon and Brent (2010), these differences likely occurred because the various criteria used by previous authors were even more liberal than our relaxed criteria. For example, in a study of the effects of temperature on diapause induction, Beards and Strong (1966) considered bugs as diapausing even in the absence of fat body hypertrophy. Other studies relied in part on characters that Spurgeon and Brent (2010) deemed irrelevant, such as atrophied seminal vesicles (Leigh 1966) and atrophied testes (Strong et al. 1970).

In addition to the effects of long-term laboratory culture of *L. hesperus* on the diapause response, behaviors related to reproduction were influenced. The propensity to mate and the proportion of multiple inseminations in lab-reared females was higher than for females originating from the field (Fig. 2). Although female *Lygus* are known to oviposit as virgins (Strong et al. 1970, Brent 2010a,b), we observed a much higher probability of oviposition during the first 10 d of adulthood for colony reared virgin females compared with bugs from the field. These changes with laboratory culture do not represent additions or deletions from the repertoire of *Lygus* behaviors, but rather shifting frequencies of the occurrence of specific behaviors. The mechanisms responsible for these shifts are presently unknown, but they are undoubtedly related to selection pressures favoring early and frequent reproduction. As such, they may represent behavioral markers of utility in assessing the similarity of cultured and native *Lygus* adults.

An additional parameter with potential value as an indicator of colony-induced selection is the occurrence of the novel 'glass bead' fat type. Although the functions and physiological implications of this fat type are unknown, its occurrence appears unrelated to

bug gender or physiological status. Still, it is apparent that this fat type becomes markedly less common in *L. hesperus* under long-term laboratory culture (Fig. 3).

Our results clearly demonstrate physiological and behavior consequences to long-term laboratory culture of *L. hesperus*. In particular, recognition of the diminished diapause response resulting from laboratory rearing may be of critical importance in designing research to better understand diapause. Our findings also highlight additional indicators of potential use for measuring the impact of laboratory selection. Significant effects were identified in both behavioral (propensity to mate and oviposit) and physiological (fat type) parameters tested. Successful development and implementation of such indicators could provide a means to ensure the availability of high-quality laboratory reared insects, and to preserve the ecological relevance of research that relies on reared insects.

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